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## Response of *Pteris vittata* to different cadmium treatments

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Reviewers' comments:

I accept scientific revision. In terms of graphics, please submit graphs with better resolution (simply made using Excel).

We have increased the quality of our graphs starting from Excel.

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**Response of *Pteris vittata* to different cadmium treatments**

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## Abstract

*Pteris vittata* is known as an arsenic hyperaccumulator but there is little information about its tolerance to cadmium and on its ability to accumulate this heavy metal. Our aim was to analyse the accumulation capacity, oxidative stress and antioxidant response of this fern after cadmium treatments. Cadmium content, main markers of oxidative stress and antioxidant response were detected in leaves of plants grown in hydroponics for both short- (5 days) and long- (15 days) term exposure to 0 (control) 60 and 100  $\mu\text{M}$   $\text{CdCl}_2$ . In leaves the concentration of cadmium and oxidative stress were parallel with the increase of cadmium exposure. In the short-term exposure antioxidant response was sufficient to contrast cadmium phytotoxicity only in 60  $\mu\text{M}$  cadmium treated plants. In the long-term exposure all treated plants, in spite of the increase in activity of some peroxide-scavenging enzymes, showed a significant increase in oxidative damage. As in the long-term stress markers were comparable in all treated plants, with no clear correlation with hydrogen peroxide content, at least part of cadmium-induced oxidative injury seems not mediated by  $\text{H}_2\text{O}_2$ . Based on our studies, *Pteris vittata*, able to uptake relatively high concentrations of cadmium, is only partially tolerant to this heavy metal.

**Keywords** Antioxidant response · Cadmium · Leaves · Oxidative stress · *Pteris vittata* · Tolerance

## Introduction

Cadmium is a non-essential heavy metal, widespread in the environment due to natural sources and human activities (Pál et al. 2006). It is considered one of the most dangerous pollutants because of its high water solubility and toxicity for most living organisms, plants included (Romero-Puertas et al. 2004). In fact this element can be absorbed by plants from soil or water and it is toxic to plant cells even at low concentrations (Gallego et al. 2012). Cadmium can cause several symptoms of toxicity such as leaf chlorosis, growth and photosynthesis inhibition (Yu et al. 2013), disturbance in water balance (Aghaz et al. 2013). Similar to other heavy metals, it is known to induce oxidative

stress (Wang et al. 2008). It has been suggested that cadmium induces oxidative stress mainly through indirect mechanisms (Wang et al. 2008) such as the interaction with the antioxidant machinery (Chaoui et al. 1997), inhibition of electron transport chain (Smeets et al. 2005) and activation of lipoxygenase with resulting lipid peroxidation (Somashenkaraiah et al. 1992). In addition, cadmium, non-redox reactive metal, is able to induce indirectly reactive oxygen species (ROS) production (Gallego et al. 2012) for example, through activation of NADPH oxidase in membranes (Gallego et al. 2012).

Reactive oxygen species, such as superoxide, hydrogen peroxide and hydroxyl radicals produced in a controlled-manner in physiological conditions, can be extremely harmful to organisms at high concentration; ROS can oxidise proteins, lipids and nucleic acids leading to alteration in cell structures and mutagenesis (Benavides et al. 2005). Apart from the damage they cause, Cd-induced ROS also exert a positive role. ROS accumulation, in fact, has been suggested as part of the signalling cascade leading to stress protection, acting as an elicitor of common stress response in plants (Mittler 2002). To counteract the damaging effects of ROS, plants have evolved an antioxidant defence machinery, including both enzymatic and non-enzymatic mechanisms. Compatible solutes, such as proline, contribute to protect plants from stress through the detoxification of reactive oxygen species, cellular osmotic adjustment, protection of membrane integrity and stabilization of enzymes/proteins, playing functions also in stress signalling (Szabados and Savouré 2009). Low molecular weight antioxidants, such as ascorbate and glutathione, can cooperate with the enzymes ascorbate peroxidase (APX) and glutathione reductase (GR) in scavenging ROS through the ascorbate-glutathione cycle (Lenher et al. 2006). Glutathione peroxidase (GPX), guaiacol peroxidase (POD) and catalase (CAT) are other important enzymes in hydrogen peroxide detoxification (Mittler 2002).

*Pteris vittata* is a perennial and fast-growing fern species, known as an arsenic hyperaccumulator (Drava et al. 2012). In As contaminated soil and water often higher contents of heavy metals such as Cd co-exist. The use of *P. vittata* for remediation or revegetation of these polluted soils depends

also on its Cd tolerance and accumulation (Xiao et al. 2008). Gupta and Devi (1994) described damage caused by Cd at different levels in the entire life cycle of *P. vittata*; in addition, Drava et al. (2012) reported the accumulation of cadmium by this fern resulting in a negative impact on plant growth. In contrast, Fayiga et al. (2004) recorded a Cd-induced stimulation of plant growth. Finally, Xiao et al. (2008) found ecotypical differences with Cd-tolerant ecotypes able to grow in polluted sites co-contaminated with Cd and As. Based on the few and contrasting findings available on the tolerance of this fern and on its ability of accumulation of Cd (Drava et al. 2012), the aim of this work was to analyse the phytotoxicity of cadmium to *P. vittata* and the tolerance of this fern to this heavy metal. As oxidative stress play a major role in metal toxicity (Sharma and Dietz 2009), oxidative damage and antioxidant defence response were studied after both short- (5 days) and long- (15 days) term exposure to different concentrations of cadmium.

## Materials and Metods

### Plant material

Spores of *Pteris vittata*, collected from mature fertile fronds, germinated within two weeks period by sowing on a mixture of potting soil (75%), and sand (25%). After four weeks, most of the germinated spores developed into gametophyte stage, fourteen weeks were needed for the development of sporophytes. The plants were watered on a daily basis or as necessary. During fern growth, the temperature in the greenhouse was 23 °C with 16/8 h light/dark photoperiod. Healthy and uniform *Pteris vittata* plants with 8 - 9 fronds were selected for the experiments. Randomly selected pinnae, hereinafter referred to as leaves, were collected along the length of fronds and immediately used or fixed in liquid nitrogen and stored at -20 °C until use.

### Cadmium treatments

The plants were acclimatized in a hydroponic system in 0.2 strength Hoagland nutrient solution for two weeks, 5 plants of *P. vittata* for each treatment were transferred into hydroponic pots supplied

with 0  $\mu\text{M}$  (control), 60  $\mu\text{M}$ , 100  $\mu\text{M}$  of  $\text{CdCl}_2$  and maintained in a greenhouse for five and fifteen days. Air supply during hydroponic cultivation was provided by an aquarium air pump in the medium solution.

#### Cadmium content determination

Fronds from five control plants and five plants treated with Cd after 5 and 15 days were, oven-dried at 60 °C for 4 days, then were digested with nitric-perchloric acid (3:1, v/v) at 100 °C. A microwave oven MLS-1200 Mega (Milestone-FKV, USA) equipped with a UV generation device was used to eliminate the interfering organic substances. Then, the measurements of Cd content were made by an atomic absorption spectrophotometer (Analyst 300; Perkin Elmer, Überlingen, Germany). For each treatment, measurements were made in triplicate.

#### Determination of water content and of relative water content

Calculations of leaf fresh weight, dry weight and moisture content were based on weights determined before and after oven drying (100 °C) of leaf samples to constant weight. Water content percentage was estimated on the fresh weight basis. Leaf relative water content (RWC; Turner 1981 with minor modifications) was calculated with the formula:

$$\text{RWC} = [(\text{FW}-\text{DW})/(\text{TW}-\text{DW})] \times 100$$

FW is fresh weight, DW is dry weight and TW is turgid weight.

Fresh weight was obtained by weighing the fresh leaves. The leaves were then immersed in water over night, blotted dry and then weighed to get the turgid weight. The leaves were then dried in an oven at 100 °C to constant weight and reweighed to obtain the dry weight.

#### Chlorophyll determination

Chlorophylls (*a*, *b* and total) and carotenoids were extracted in 80% acetone and determined according to Hassanzadeh et al. (2009) and to Lichtenthaler (1987) respectively. Fresh leaves were homogenised and after centrifugation, for 10 min at 6,000g at 4 °C, the extracts were centrifuged

and the supernatants were collected. The pellets were resuspended and extracted with 80% acetone until they resulted colourless. The collected supernatants were read spectrophotometrically at 645, 663 and 470 nm and pigment contents were expressed as  $\text{mg g}^{-1}$  DW.

#### Hydrogen peroxide and lipid peroxidation

Hydrogen peroxide content of leaves was determined according to Jana and Choudhuri (1982) spectrophotometrically at 410 nm, using titanium chloride in  $\text{H}_2\text{SO}_4$  for peroxide detection. The amount of  $\text{H}_2\text{O}_2$  in the extracts, calculated from a standard curve, was expressed as  $\mu\text{mol g}^{-1}$  DW.

The amount of lipid peroxidation products in leaves was estimated by determining the malonyldialdehyde (MDA) content in the leaves according to Hartley-Whitaker et al. (2001) with minor modifications as in Spanò et al. (2007). Freeze-dried leaves were powdered and mixed with TBA reagent (10% w/v trichloroacetic acid + 0.25% w/v thiobarbituric acid), heated ( $95^\circ\text{C}$ ), cooled and centrifuged. Malonyldialdehyde ( $155 \text{ mM}^{-1} \text{ cm}^{-1}$  extinction coefficient) content was measured as specific absorbance at 532 nm and by subtracting the non-specific absorbance at 600 nm (De Vos et al. 1989) and expressed as TBARS (TBA-reactive materials) in  $\text{nmol g}^{-1}$  DW.

#### Electrolytic conductivity method for membrane damage estimation

Membrane damage was estimated as in Spanò et al. (2002) with minor modifications as in Spanò et al. (2013). Segments of leaves (0.5 cm, cut 0.5 cm from the tip), after a short washing, were incubated in deionised water, evacuated for 30 min, and allowed stirring for 22 h at  $4^\circ\text{C}$ . The conductivity of the aqueous solution was measured with a Jeenway 4310 Conductivity Meter at  $25^\circ\text{C}$ . Conductivity was also detected at  $25^\circ\text{C}$  after boiling the test tube in a water bath for 1 h. The percentage injury was calculated according to as a percentage of membrane damage using the formula:

$$(C_1 - C_w) / (C_2 - C_w) \times 100$$



$C_1$  is electro-conductance value of samples at the first measurement,  $C_2$  is electro-conductance value after boiling and  $C_w$  is electro-conductance value of deionised water.

#### Extraction and determination of proline, ascorbate, and glutathione

Proline concentration was determined according to the method of Bates et al. (1973) with minor modifications, as in Spanò et al. (2013). Leaf tissue was homogenized with 3% sulfosalicylic acid. The supernatant was incubated with glacial acetic acid and ninhydrin reagent (1:1:1) and boiled in a water bath at 100 °C for 60 min. After cooling the reaction mixture, toluene was added, and the absorbance of toluene phase was read at 520 nm. Calculations were made on the base of a standard curve and proline content was expressed as  $\mu\text{mol g}^{-1}$  DW.

Ascorbate (AsA) and dehydroascorbate (DHA) extraction and determination were performed according to Kampfenkel et al. (1995) with minor modifications. Total ascorbate (AsA + DHA) was determined at 525 nm after reduction of DHA to AsA by dithiothreitol. Dehydroascorbate content was estimated on the basis of the difference between total ascorbate and AsA value. Calculations were made on the base of a standard curve and ascorbate content was expressed as  $\text{mg g}^{-1}$  DW.

Glutathione was extracted and determined according to Gossett et al. (1994). Total glutathione (GSH + GSSG) was determined by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase recycling procedure. The reaction was monitored as the rate of change in absorbance at 412 nm and GSSG was determined after removal of GSH from the sample extract by 2-vinylpyridine derivatisation. Calculations were made on the basis of a standard curve and content was expressed as  $\text{nmol g}^{-1}$  DW.

#### Enzyme extraction and assays

Leaves were ground in liquid nitrogen with a mortar and pestle. Extraction was made according to Spanò et al. (2013) at 4 °C. For APX, 2 mM ascorbate was added to the extraction medium, and for GR the supernatant was desalted on a Sephadex G-25 column. The extracts were stored in liquid

nitrogen until use.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) with modifications. Enzyme activity was assayed from the decrease in absorbance at 290 nm (absorbance coefficient of  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ) as ascorbate was oxidised, one unit of the enzyme oxidising  $1.0 \mu\text{mole}$  of -ascorbate to dehydroascorbate per min. Enzymatic extract contained  $25 \mu\text{g protein ml}^{-1}$ . Correction was made for the low, non enzymatic oxidation of ascorbate by hydrogen peroxide (blank).

Glutathione reductase (GR, EC 1.6.4.2) activity was determined as described by Rao et al. (1995) following the oxidation of NADPH at 340 nm (extinction coefficient  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ), one unit reducing  $1.0 \mu\text{mole}$  of oxidised glutathione per min. Enzymatic extract contained  $25 \mu\text{g protein ml}^{-1}$ . A correction for the non-enzymatic oxidation of NADPH was carried out in the absence of enzyme extract (blank).

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to Navari-Izzo et al. (1997) by coupling its reaction with that of GR, using as substrate  $0.45 \text{ mM H}_2\text{O}_2$ . The activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Enzymatic extract contained  $25 \mu\text{g protein ml}^{-1}$ .

Catalase (CAT, EC 1.11.1.6) activity was determined as described by Aebi (1984). Enzymatic extract contained  $25 \mu\text{g protein ml}^{-1}$ . A blank containing only the enzymatic solution was made. Specific activity was calculated from the  $23.5 \text{ mM}^{-1} \text{ cm}^{-1}$  extinction coefficient, one unit decomposing  $1.0 \mu\text{mole}$  of hydrogen peroxide to oxygen and water per min.

Guaiacol peroxidase (POD, EC 1.11.1.7) activity was determined as described by Arezky et al. (2001) using as substrate 1% guaiacol. Enzymatic extract contained  $25 \mu\text{g protein ml}^{-1}$ . Enzymatic activity was determined following guaiacol oxidation by  $\text{H}_2\text{O}_2$  (extinction coefficient  $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ) at 470 nm, one unit oxidising  $1.0 \mu\text{mole}$  guaiacol per min.

All enzymatic activities were determined at  $25^\circ\text{C}$  and expressed as enzyme units (U) per g soluble protein. The total soluble protein content was measured according to Bradford (1976), using

BSA as standard.

## Statistical analysis

The data were based on three replicates from three independent experiments. All data were analysed by one-way analysis of variance (ANOVA) and the mean differences were compared using the Student Newman Keuls test.

## Results

### Cadmium content

In leaves the accumulation of cadmium was parallel with the increase of cadmium concentration in growth medium and of the time of treatment (Table 1). After 5 days only plants treated with the higher concentration showed a concentration of this heavy metal in the range 5-10  $\mu\text{g g}^{-1}$  DW. By day 15, in leaves from plants treated with 100  $\mu\text{M}$  cadmium, the concentration of this toxic metal reached values higher than 150 ppm.

### Water status and chlorophyll content

Water content was not significantly different in all samples (Table 1). All treated samples showed a significant decrease in RWC value at 15 days of treatment. The ratio of fresh weight to dry weight, not significantly different among the samples at 5 days (about 6), decreased at 15 days in Cd-treated samples reaching the minimum value in 100  $\mu\text{M}$  Cd-treated plants (4,88). While the content of pigments (Table 1) was not significantly different at 5 days in all samples, after 15 days only total chlorophyll was significantly lower in Cd-treated samples. Chl*a*/Chl*b* ratio was significantly lower only in 100  $\mu\text{M}$  Cd-treated plants.

### Oxidative stress evaluation

ROS production was monitored by measuring the concentration of hydrogen peroxide. The content

of this signalling molecule (Fig. 1) was significantly higher in the plants treated with 100  $\mu$ M Cd than in other samples both at 5 days and at 15 days. Lipid peroxidation (TBARS, Fig. 1) was consistently higher in treated plants than in control plants both after 5 and after 15 days. However, after 5 days membrane damage assessed by the electrolytic conductivity method (Fig. 1) was significantly higher only in the maximum Cd concentration, while after 15 days of treatment all treated plants showed the damage value significantly higher than in control plants.

#### Proline and non enzymatic antioxidants

Proline content (Table 2) was significantly higher in Cd-treated than in control plants, both after 5 and after 15 days of treatment. There was a strong decrease in its content in Cd-treated plants from 5 to 15 days of treatment. Antioxidant content was assessed by monitoring variations in ascorbate and glutathione. Total ascorbate (AsA + DHA, (a reduced form + an oxidised form, respectively), Table 2) was significantly higher in treated plants after 5 days, while at 15 days in 60  $\mu$ M Cd-treated plants there was a strong reduction (to about one half) of the content of this low molecular weight antioxidant. Interestingly, just these plants showed the highest reducing power of AsA/DHA couple (Table 2). Total glutathione (GSH + GSSG, (a reduced form + an oxidised form, respectively), Table 2) was higher in 5 days treated plants than in control ones and the highest value was detected in 100  $\mu$ M Cd plants while after 15 days it was not significantly different between treated and control plants. Reducing power of GSH/GSSG couple was significantly lower in 100  $\mu$ M Cd plants after 5 days, while at 15 days both treated samples had a reducing power that was significantly lower than in control plants.

#### Antioxidant enzymes

Results of enzymatic assays are shown in Fig. 2. After 5 days of Cd treatment the activities of ascorbate peroxidase (APX), guaiacol peroxidase (POD) and catalase (CAT) were significantly lower in Cd-treated plants. In particular POD activity reached the minimum value in 100  $\mu$ M Cd-

treated plants. Unlike than other enzymes the glutathione peroxidase (GPX) showed significantly higher activities in stressed plants. After 15 days of treatment APX and CAT activities were significantly lower in 100  $\mu\text{M}$  Cd plants. Instead of that the activity of POD was higher in stressed than in control plants with a maximum in 60  $\mu\text{M}$  Cd plants. Glutathione peroxidase activity was not significantly different among the three samples. GR activity did not differ significantly after 5 days nor after 15 days among control and stressed plants.

## Discussion

*Pteris vittata* has been reported as a natural hyperaccumulator plant for arsenic (Ma et al. 2001), but not for heavy metals such as mercury (Chen et al. 2009). Our focus was to assess performance and antioxidant response of this plant to cadmium stress. Cadmium able to cause several symptoms of phytotoxicity (Sanità di Toppi and Gabbrielli 1999), can induce oxidative stress by the generation of ROS able to cause damage to macromolecules and cell structures.

### Cadmium content of leaf and oxidative stress

In *Pteris vittata* after five days of treatment only leaves of plants treated with the highest Cd concentration showed a content of this heavy metal in the range 5-10  $\mu\text{g g}^{-1}$  DW, considered toxic to most plants (White and Brown 2010). In this condition leaching and lipid peroxidation (indirectly indicating the membrane damage), the content of hydrogen peroxide and proline, stress markers, were significantly higher than in control plants. Despite the leaf cadmium content was below the threshold of toxicity, also the plants treated with the lower concentration of this metal showed some signs of stress, such as high lipid peroxidation and proline content, probably due to the presence of higher concentration of Cd in the underground part of the plant (data not shown) whose effects can be overtaken on the rest of the plant. In 100  $\mu\text{M}$  Cd plants only lipid peroxidation further increased after 15 days of treatment, showing that the other stress parameters reached their maximum value already after the first period of treatment. On the contrary in 60  $\mu\text{M}$  Cd plants both leaching and

lipid peroxidation further increased after 15 days, reaching values not significantly different from plants treated with the maximum Cd concentration. Interestingly, there was not a correlation between the hydrogen peroxide content and the membrane damage level assessed as lipid peroxidation and this seems to confirm that cadmium can cause damage by an indirect induction of oxidative stress, not ROS-mediated. As a matter of fact, a lipid peroxidation induced by the Cd-mediated activation of lipoxygenase has been reported (Chaoui et al. 1997).

#### Relative water content and pigments

Despite some signs of stress were evident after 5 days of treatment, a significant decrease in RWC and in FW/DW ratio, generally accepted as an indicator of phytotoxicity of heavy metal stress (Su et al. 2005, J6cs6k et al. 2008), was recorded in treated plants after 15 days. In Cd-treated plants disturbance of water balance, already reported in literature (Benavides et al. 2005), was associated with a significant decrease in total chlorophyll content, another effect of Cd toxicity (Sanit6 di Toppi and Gabbrielli, 1999). However a significant decrease in Chla/Chlb ratio, another stress indicator (Rout and Shaw 2001), was only recorded in 100  $\mu$ M Cd plants, underlining the different effects of used Cd concentrations on *P. vittata*. Carotenoids, often found increased under stress condition (Munne-Bosch and Alegre 2000), did not significantly differ among the different treatments, in accordance to a minor effect of cadmium on this pigment group (P6l et al. 2006).

#### Proline and antioxidant molecules

Changes in proline content, characterised by a strong decrease in treated samples from 5 to 15 days, are rather interesting. As commonly accepted, cadmium has a serious negative impact on nitrogen uptake and distribution (P6l et al. 2006), what could impair the accumulation of nitrogen-containing defence molecules, such as proline. The decrease in proline content in 15-day treated plants could be considered as a way to recover nitrogen for the synthesis of other amino acids (Dandekar and Uratsu 1988). As it is commonly accepted that proline plays a protective role against oxidative stress induced by Cd (Hossain et al. 2010), the decrease of its content in the long period can be

considered a symptom of the only partial tolerance of *P. vittata* to this heavy metal.

Ascorbate is one of the low molecular weight antioxidants involved in plant defence system (Mittler 2002) and in the short period *P. vittata* was able to efficiently regulate the pool of this molecule. In the long period however, in accordance with data on mercury exposure (Chen et al. 2009), ascorbate pool significantly decreased in 60  $\mu$ M Cd-treated plants. On the other hand there was a concentration-dependent increase in AsA/DHA ratio, generally reported as an indicator of a low sensitivity to stress (Gupta et al. 1999). This ratio was always significantly higher in treated plants than in control plants, in particular after 15 days of treatment, and it was a signal of a good capacity of *P. vittata* to maintain a high reducing power under cadmium treatment. The strong increase in glutathione pool after 5 days strictly depended on cadmium concentration and is a further confirmation of the importance of this sulphur-containing molecule in stress response (Gallego et al. 2012). The significant depletion of glutathione concentration in the long-term treatment, particularly at the expense of the reduced form, could be in part explained considering the increase in production of phytochelatin in the presence of high concentrations of cadmium (Lux et al. 2011) and the role of GSH in phytochelatin biosynthesis (Ederli et al. 2004). In fact these molecules have been given a role in detoxification of elements like arsenic and cadmium in Indian mustard (Pickering et al. 2000; Zhu et al. 1999) and copper in bean (Gupta et al. 1998). The decrease in reducing power of GSH/GSSG couple in treated samples was generally proportional to the increase in reducing power of AsA/DHA couple, showing the importance of glutathione in AsA-GSH cycle, the major pathway in protection against cadmium stress (Smeets et al. 2009).

#### Antioxidant enzymes

Our results clearly indicated an involvement of antioxidant enzymes in stress defence. In the short-term treatment, when enzymatic activity was generally lower in Cd-treated than in control plants, among the enzymes studied, GPX seems to contribute in hydrogen peroxide detoxification, that only in 60  $\mu$ M Cd-treated plants was not significantly different from control plants. The decrease in

GPX after 15 days treatment, is in 60  $\mu$ M Cd-treated plants partially compensated by the increase in activity of CAT and APX reaching values of activity of control plants. In particular POD showed a significant stimulation in the long-term Cd treatment, as it was already reported for *Phragmites australis* (Ederli et al. 2004).

## Conclusions

In conclusion, cadmium induced oxidative damage in *Pteris vittata* that only in the short-term was able to tolerate this heavy metal by activation of an adequate antioxidant response based mainly on proline, ascorbate, glutathione and the scavenging contribution of GPX. This also when the Cd content was in the range considered as toxic to most plants. In the long-term treatment, the 100  $\mu$ M Cd-treated plants had in leaves high content of cadmium, however significantly lower than those detected in Cd hyperaccumulators (Kováčik, 2013), but showed clear signs of damage accompanied by an increase of all tested markers of oxidative damage and a failure to relieve the inhibition of most of the antioxidant enzymes. As in the long-term treatment the values of stress markers were comparable in all treated plants, with no clear correlation with hydrogen peroxide content, we must conclude that at least part of the oxidative injury induced by cadmium is not mediated by hydrogen peroxide.

**Author contribution** All the authors conceived, designed and performed the experiments and wrote the paper.



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#### Figure captions

**Fig. 1** Hydrogen peroxide content, a; lipid peroxidation measured by determining thiobarbituric acid reactive substances (TBARS), b; membrane damage assessed by electrolytic conductivity method, c in leaves of *P. vittata* plants treated with 0 (control), 60 and 100  $\mu$ M Cd for 5 and 15 days. Values are means of triplicate and vertical bars indicate SE. Different letters denote significant differences at  $P < 0.05$ .

**Fig. 2** Activity of ascorbate peroxidase (APX), a; glutathione reductase (GR), b; glutathione peroxidase (GPX), c; catalase (CAT), d; guaiacol peroxidase (POD), e, in leaves of *P. vittata* plants treated with 0 (control), 60 and 100  $\mu$ M Cd for 5 and 15 days. Values are means of triplicate and vertical bars indicate SE. Different letters denote significant differences at  $P < 0.05$ .

Figure 1  
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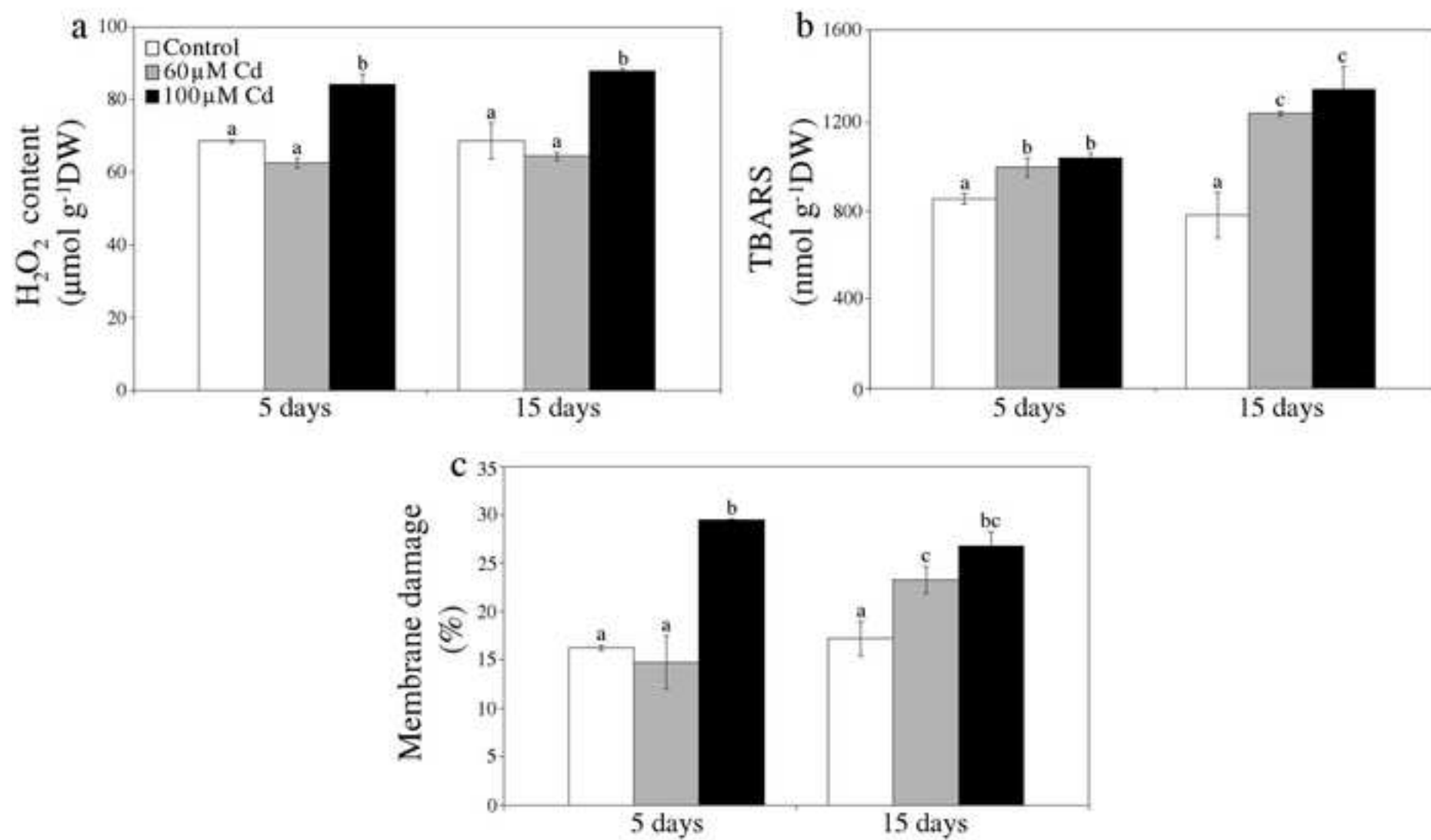
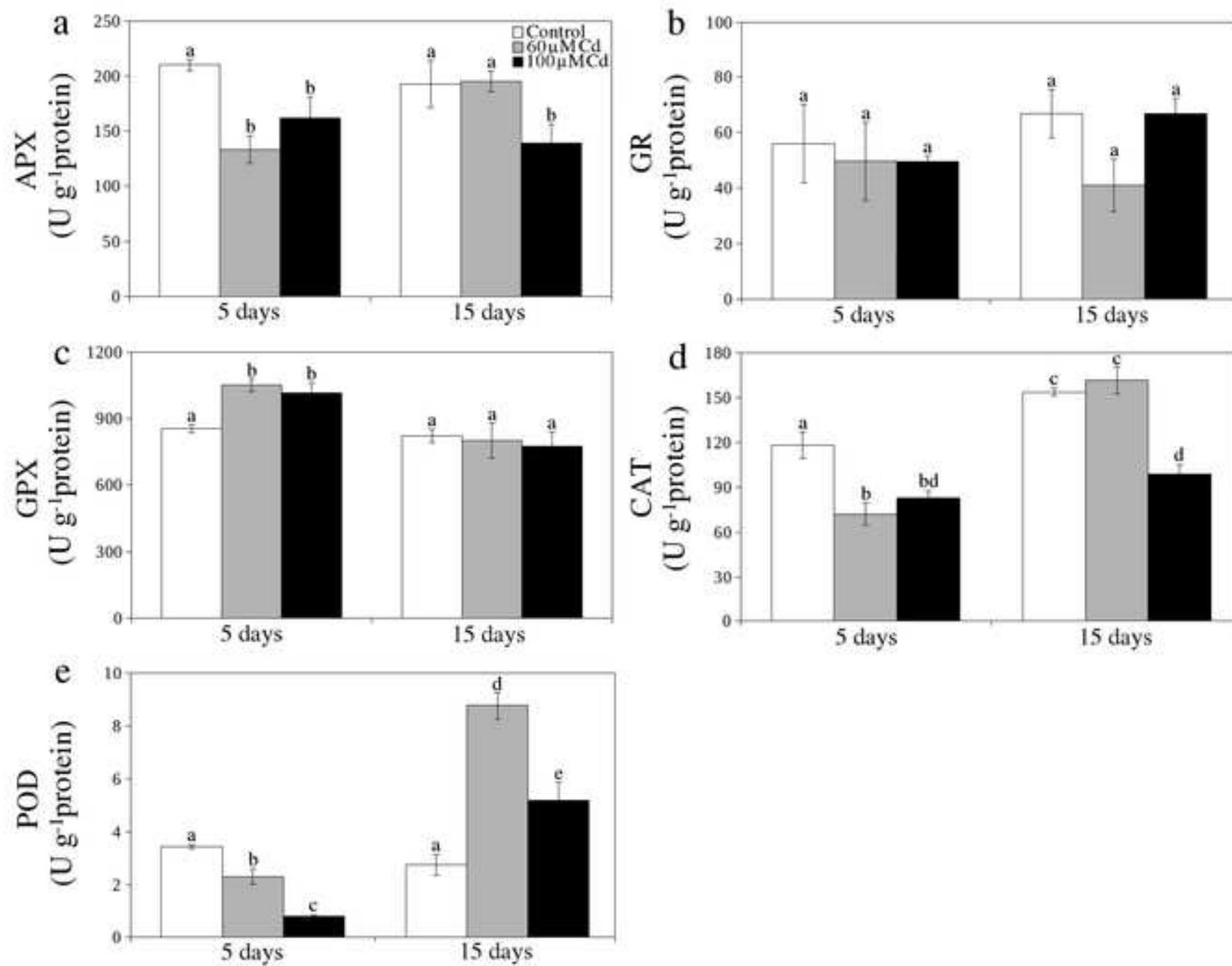


Figure 2  
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**Table 1** Cadmium content, water content, relative water content (RWC) and pigment content of leaves of *P. vittata* plants treated with 0 (control), 60 and 100 µM Cd for 5 and 15 days.

	5 days			15 days		
	Control	60 µM Cd	100 µM Cd	Control	60 µM Cd	100 µM Cd
Cd content of leaf (µg g <sup>-1</sup> DW)	0.016±0.001e	0.326±0.002d	7.998±0.007c	0.014±0.001e	72.040±0.002b	159.150±0.001a
RWC (%)	90.83±1.01a	87.00±2.45a	89.00±0.33a	87.00±1.20a	77.50±3.67b	75.90±1.80b
Total chlorophyll (mg g <sup>-1</sup> DW)	18.03±0.53a	15.20±2.03a	15.75±2.40a	19.01±0.25a	13.03±1.37b	13.90±1.62b
Chla/Chlb	2.18±0.01a	2.18±0.01a	2.11±0.00a	2.22±0.02a	2.02±0.02a	1.67±0.00b
Carotenoids (mg g <sup>-1</sup> DW)	2.54±0.40a	2.27±0.19a	2.47±0.29a	2.59±0.16a	2.03±0.25a	2.07±0.13a

Values are means of triplicate ± SE. Different letters denote significant differences at P < 0.05

**Table 2** Contents of proline, ascorbate: total, reduced (AsA) and oxidised (DHA), glutathione: total reduced (GSH) and oxidised (GSSG) in leaves of *P. vittata* plants treated with 0 (control), 60 and 100 µM Cd for 5 and 15 days.

	5 days			15 days		
	Control	60 µM Cd	100 µM Cd	Control	60 µM Cd	100 µM Cd
Proline (µmol g <sup>-1</sup> DW)	7.60±1.11d	23.43±0.35a	20.13±0.97b	6.66±1.33d	13.88±0.01c	15.61±0.01c
AsA (mg g <sup>-1</sup> DW)	0.31±0.01d	1.16±0.01a	0.82±0.03b	0.61±0.06c	0.62±0.06c	1.08±0.02a
DHA (mg g <sup>-1</sup> DW)	0.54±0.02ab	0.54±0.01ab	0.65±0.04a	0.65±0.01a	0.21±0.06c	0.42±0.03b
Total ascorbate (mg g <sup>-1</sup> DW)	0.85±0.01d	1.70±0.02a	1.47±0.04b	1.26±0.04c	0.83±0.01d	1.50±0.01b
AsA/DHA	0.57±0.00e	2.15±0.03b	1.26±0.00c	0.94±0.01d	2.95±0.01a	2.57±0.00b
GSH (nmol g <sup>-1</sup> DW)	53.65±2.39c	101.52±8.08b	203.33±19.84a	89.11±4.92bc	53.88±3.81c	51.93±8.74c
GSSG (nmol g <sup>-1</sup> DW)	10.02±1.19d	20.74±3.43c	89.29±2.35a	26.15±3.37c	27.27±0.67c	42.28±1.68b
Total glutathione (nmol g <sup>-1</sup> DW)	73.69±0.00c	143.00±3.17b	381.92±15.16a	141.42±11.68b	108.42±4.74b	136.5±22.86b
GSH/GSSG	5.35±0.03a	4.89±0.01b	2.28±0.01d	3.41±0.02c	1.97±0.03d	1.23±0.00e

Values are means of triplicate ± SE. Different letters denote significant differences at P < 0.05

### **Author contribution**

All the authors of this manuscript equally contributed to conceive, design and perform the experiments and to wrote the paper.